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EGGS OF *PALAEMON MACRODACTYLUS*: III. INFECTION BY THE FUNGUS, *LAGENIDIUM CALLINECTES*

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ABSTRACT

Eggs of the estuarine shrimp, *Palaemon macrodactylus*, were studied under various conditions to determine their susceptibility to a known fungal pathogen, *Lagenidium callinectes*. Eggs exhibited no signs of fungal infection under normal conditions, but were infected when detached from the female or when the female's first pereiopods ("cleaning chelipeds") were excised. This implied that preening or egg removal by the first pereiopods halted infection or obviated detection of the fungus. Nutrient addition increased the number of infections and decreased the time to infection. Fertilized eggs were less susceptible to infection than unfertilized, probably the result of the hardened fertilization membrane restricting fungal penetration. Similarly, older embryos with progressively hardened coats were also less susceptible. Defense may have also been provided by antifungal bacteria isolated from embryo surfaces. Two of these epizooic bacteria belong to the marine genus, *Alteromonas*, and release extracellular enzymes, including lipase and chitinase. Both bacteria were found to inhibit a variety of pathogenic fungi, including six strains of *Lagenidium callinectes*, two strains of *Halipthoros milfordensis* and a freshwater *Saprolegnia* species.

INTRODUCTION

Two phycomycetous fungi have repeatedly been found to infect crustaceans in nature and in aquaculture situations. Couch (1942) first described *Lagenidium callinectes* parasitizing eggs of the blue crab, *Callinectes sapidus*. The fungus has subsequently been found pathogenic to penaeid shrimp larvae (Cook, 1971; Lightner and Fontaine, 1973), cultured larvae of the American lobster, *Homarus americanus* (Nilson *et al.*, 1976), and in zoeae of the Dungeness crab, *Cancer magister* (Armstrong *et al.*, 1976). A complete description of this phycomycete isolated from blue crab eggs has been provided by Bland and Amerson (1973). The pathogenic effects of another fungus, *Haliphthoros milfordensis*, were described (Fisher *et al.*, 1975) on *Homarus americanus* and *Homarus gammarus* postlarvae in aquaculture systems. *H. milfordensis* was later found to infect adult penaeid shrimp, ova and larvae of the brine shrimp, *Artemia salina*, and eggs of *C. sapidus* (Tharp and Bland, 1977). An unidentified fungus was also reported to infect detached eggs of *Palaemon idae* (Aiyer, 1949).

Chemical control of these destructive pathogens in aquaculture systems has been investigated by a variety of authors (Delves-Broughton, 1974; Armstrong *et al.*, 1976; Bland *et al.*, 1976; Fisher *et al.*, 1976; Abrahams and Brown, 1977). Antibiotic control has not been investigated and, historically, there have been very few references to microbial activity against fungi (Horikoshi and Iida, 1958). There are, however, several marine bacteria known to produce substances toxic to other bac-

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teria. Four species of the genus *Alteromonas* produce antibiotics against Gram-positive and some Gram-negative bacteria: *A. rubra* (Gauthier, 1976; Ballester *et al.*, 1977), *A. luteoviolaceus* (Anderson *et al.*, 1974; Gauthier and Flatau, 1976), *A. citrea* (Gauthier, 1977), and *A. aurantia* (Gauthier and Breittmayer, 1979). Several strains of other *Alteromonas* species (*A. haloplanktis, A. undina,* and *A. espejiana*) have been found off the coast of northern California (Chan *et al.*, 1978), but none were tested for antibiotic activity. Burkholder *et al.* (1966) describe a bacterium, *Pseudomonas bromoutilis* strain 287, that matches *A. haloplanktis* in all the recorded metabolic characteristics and does produce an antibiotic substance. None of these have been tested against fungi.

Palaemon macrodactylus, an estuarine shrimp introduced to the San Francisco Bay area from the Orient around 1954 (Newman, 1963), has not been tested for susceptibility to fungal infections. This study describes the infection of laboratory-held adults and brooding embryos of the shrimp *P. macrodactylus* by the phycomycete *Lagenidium callinectes.* It also examines susceptibility and potential mechanisms of protection against infection, including the release of an antifungal substance by bacteria (*Alteromonas*) isolated from embryonic surfaces. The term "egg" is used in a general sense to mean fertilized egg, unfertilized egg, or both. Where appropriate, specific designations will be "unfertilized egg," and "fertilized egg" or "embryo."

Materials and Methods

Egg susceptibility to fungal infection

Adult shrimp, *P. macrodactylus,* were held in a closed recirculating system and maintained as described previously (Fisher, 1983). Infective fungal propagules for inoculation experiments were obtained in the following manner: the fungus, isolated from previously infected eggs and designated L-Pm, was cultured on dilute Difco Marine Agar 2216 (1/3 MA). An agar slab with bacteria-free fungal hyphae was then transferred to dilute Difco Marine Broth (1/3 MB). After 2–3 days in 1/3 MB, hyphae grew out from the agar slab, and vigorous shaking for 10–15 seconds broke off fragments that could initiate new growth. Concentrations of these infective propagules were determined by plating 0.1 ml broth on 1/3 MA. Eggs infected with the fungus were fixed in 2% glutaraldehyde in a standard 0.1 *M* phosphate buffer at pH 7.6 and embedded in an epoxy medium (Spurr, 1969). Thick sections (0.5 μm) were stained with borate buffered toluidine blue (Dewel and Clark, 1972).

Two clusters of externally brooding 1-day-old embryos were detached from an ovigerous female and rinsed in sterile dilute sea water (sterile 1/3 SW). The clusters were suspended by thread into aerated 125 ml Erlenmeyer flasks containing 100 ml sterile 1/3 SW. One flask was inoculated with a 1 ml fungal suspension containing approximately 210 propagules and the embryos of both clusters were monitored for signs of infection for 7 days.

The first or third pair of pereiopods were excised from two mated females extruding eggs on the same day. Excision occurred 3 days after oviposition and embryos were examined intermittently for fungal infection until hatching. The first and third pereiopods correspond respectively to the "cleaning chelipeds" and "first walking legs" described by Bauer (1979). In similar experiments, the first pereiopods were excised from two other mated females, 1 day and 6 days after oviposition, and one unmated female whose first pereiopods were excised 1 day after extrusion of unfertilized eggs.

A series of tests were conducted to determine the effect of fertilization on egg susceptibility to L-Pm. In six separate experiments, 1-day-old unfertilized egg clusters were suspended in the same aerated flask with 1-day-old fertilized egg clusters and a fungal inoculum. To determine the effect of brooding time, embryos aged 2, 4, 7, 10 and 12 days were suspended and compared in three separate trials. Fungal inocula for these experiments ranged from 30–200 infective propagules and, in some cases, 1/3 MB was added as nutrient at a concentration of 5%.

Characteristics of the fungus, L-Pm

Samples of L-Pm were sent to Dr. C. Bland, East Carolina University, Greenville, North Carolina for examination. L-Pm was also plated by agar slab transfer to different agar media where radial growth rates were compared to six isolates of *Lagenidium callinectes* and two isolates of *Haliphthoros milfordensis*, all obtained through the courtesy of C. Bland and L. Crisp (East Carolina University), and one *Saprolegnia sp.* isolated from the white sturgeon, *Acipenser transmontanus*. Designations for these isolates are reported in Table II. The media used for fungal growth tests were 1/3 MA, shrimp infusion agar (SIA), and corn meal extract agar (CMA). SIA was prepared by autoclaving 10–15 medium-sized shrimp (*Crangon*) in 1 liter of 1/3 SW, straining, and adding 0.2% glucose. CMA was prepared by boiling 25 g of Albers yellow corn meal for 3 minutes in distilled water, filtering through 2 layers of cheesecloth, then adding distilled water to 1 liter. For 1/3 SW-CMA and 1/2 SW-CMA, appropriate dilutions of sea water were substituted for the distilled water. NaCl and KCl were added to CMA media at concentrations of 1% and 2.5% to determine gross salt and ion optima. In one medium, 1/2 SW-CMA was supplemented with 0.3% glucose (1/2 SW-CMAG). All media contained 2% agar. Agar slabs of the various fungi were plated on these media and growth rates measured over periods of 2–25 days.

Antagonistic effects of bacteria

Embryos from 15 *P. macrodactylus* females collected from the Petaluma River on 16 June 1981 were sampled for bacteria on 1/3 MA (Fisher, 1983), and 177 bacterial colonies, the total number from duplicate plates at the 10^{-2} dilution level, were re-cultured on the peripheries of 1/3 MA plates. Agar slabs of growing fungus (L-Pm) were placed in the center of the plates, and inhibition of growth was monitored as hyphae approached the individual bacterial colonies.

Two bacterial types, both isolated from embryos of females held in the laboratory system, maintained a large zone of inhibition when plated with L-Pm. One isolate, designated I-2, had been isolated from untreated control eggs in an unpublished experiment where eggs treated with penicillin had become infected with fungus. The other isolate, designated I-13, was isolated from eggs during routine monitoring. Inocula of I-2 and I-13 were added to a test tube containing 1/3 MB and an agar slab of growing L-Pm. Control tubes received the fungal slab only and after 1–2 days, fungal growth in the tubes was compared.

Three fungal slabs were transferred to each of two, 0.45 μm-pore size, membranes laying on dehydrated nutrient pads in sterile petri dishes (Nalgene Nutrient Pad Kit). The nutrient pad in one dish was hydrated with sterile 1/3 SW while the other was hydrated with an inoculum of bacterial isolate I-2. The bacteria were separated from the fungal slab by the membrane. For three successive days, one fungal slab was removed from each membrane and transferred to a 1/3 MA plate where growth was monitored. Fungus growing from the edge of the agar slab was trimmed away

prior to transfer and tests were made to insure against possible bacterial leakage across the 0.45 μm membrane.

The effect of the antifungal bacteria on L-Pm was observed on whole-mount light microscopy, thick sections, and by scanning electron microscopy.

Characteristics of the bacteria

Bacterial isolates I-2 and I-13 were tested for Na^+ requirement, fermentation of glucose, sucrose, and fructose, production of the extracellular enzymes alginase, amylase, chitinase, gelatinase and lipase, and utilization of carbon sources according to the methods of Baumann and Baumann (1981). Flagella stains followed the method of Mayfield and Inniss (1977).

A variety of fungi were tested against antifungal bacteria by plating fungal slabs on agar and allowing time for growth to become well established, then streaking the bacteria 10–20 mm away from the growing hyphae. Inhibition was determined by comparing hyphae distance from the bacteria. The fungi involved and agar media used were previously described. The activity of various *Alteromonas* species were tested in a similar manner against the fungi L-F2, L-Pm, and S-At, all on 1/3 MA. The bacteria, obtained through the courtesy of Drs. P. Baumann and L. Baumann (University of California, Davis), included *A. macleodii* strains 113 and 107 (Type strain, ATCC 27126), *A. haloplanktis* strains 121 and 215 (Type strain, ATCC 14393), *A. espejiana* strain 261 (Type strain, ATCC 29659), *A. undina* strain 272 (Type strain, ATCC 29660), *A. communis* strain 8 (Type strain, ATCC 27118) and *A. vaga* strain 40 (Type strain, ATCC 27119).

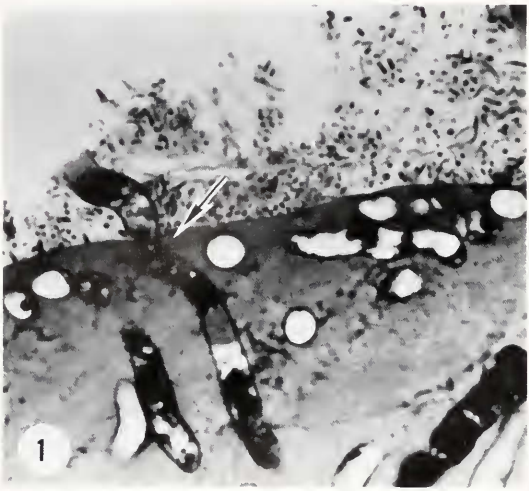
Results

Egg susceptibility to fungal infection

In all cases, embryos infected by *L. callinectes* neither survived nor hatched. Embryos suspended in a flask with fungal propagules were infected by the fungus on the third day following inoculation. The control embryos, detached from the same female but suspended in a flask without fungal propagules, showed no signs of infection after 7 days of observation. Fungus infecting unfertilized eggs penetrated the egg investment coats and totally invaded the internal milieu, destroying the otherwise apparent globular yolk structure (Fig. 1). Penetration of the egg coats appeared to cause a constriction of the fungal hyphae.

Embryos brooding on females with excised first pereiopods (cleaning chelipeds) became infected and died in all trials (Table I) whereas no dead or infected embryos were observed in the clutch of the female with her third pereiopods (walking legs) excised. No infected or dead eggs were observed in any other attached clutches throughout these studies. Excision of the first pereiopod of an unmated female (Table I, Experiment 4) allowed unfertilized eggs to remain attached for longer than 3 days, but over 80% of these eggs were infected by 5 days. All dead eggs and embryos plated on 1/3 MA showed growth of a fungus with the characteristics of L-Pm.

In all cases tested, unfertilized egg clusters were more susceptible to fungal infection than fertilized (Fig. 2). They were first to become infected and infection of all the eggs in the cluster occurred within 1 day of its first appearance on a single egg. Infection of fertilized eggs appeared 1–2 days after infection of unfertilized eggs and progressed more slowly through the cluster, requiring 3 days or longer to infect all the embryos. Age of the embryo did not consistently alter its susceptibility to the fungus, however the characteristics of the disease on mature embryos resembled



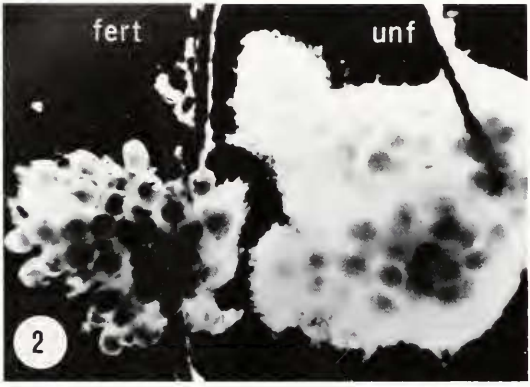


FIGURE 1. *L. callinectes* is constricted as it penetrates the outer egg layers (arrow) of this unfertilized egg. Fungal hyphae invade the internal milieu and destroy the otherwise globular ooplasm. Toluidine blue stain, 1,700×.

FIGURE 2. Unfertilized eggs were engulfed by fungal hyphae after three days incubation with L-Pm propagules while fertilized eggs suspended in the same flask were less susceptible. Even after five days, half the fertilized eggs remained uninfected.

FIGURES 3 and 4. The fungal hyphae on unfertilized eggs protruded directly outward from the egg surface whereas hyphae on fertilized eggs appeared to form a mat around the egg surface and the embryos may have been slowly asphyxiated rather than parasitized.

infestation rather than infection. Instead of penetrating each embryo individually (where hyphae could be seen protruding from the embryo surface as in Figure 3), a hyphal mat grew around the cluster and appeared to wrap the embryos (Fig. 4). First appearance of the fungus varied from 1–3 days for unfertilized eggs. Inoculated samples usually required 3 days for first appearance unless nutrient was added, which reduced the time to infection for both eggs and embryos.

Characteristics of the fungus, L-Pm

The fungus L-Pm was determined to be *Lagenidiaceous* by virtue of its extensively branched, sparingly septate hyphae of 6–10 μm cross-sectional diameter. Its

TABLE I

Fungal infection of attached fertilized and unfertilized eggs after excision of the female's first pereiopods, the "cleaning chelipeds"

Experiment number	Age (d)	Sample size (n)	% Infected
1. Fertilized	t_0* = 3	—	—
	5	25	3.8
	7	27	25.9
2. Fertilized	t_0 = 6	—	—
	10	24	4.0
	12	80	37.5
3. Fertilized	t_0 = 1	—	—
	5	30	0.0
	10	75	33.0
	12	95	84.2
4. Unfertilized	t_0 = 1	—	—
	5	17	82.4

* Embryonic age at time of excision is designated t_0.

No fungal infection was ever encountered on eggs attached to females with intact cleaning chelipeds. All infections were fatal.

radial growth rate on agar plates of different media and salt concentrations is shown in Table II. It compares most favorably with *L. callinectes* isolate L-F2, which was previously isolated from northern California waters. Both show rapid growth at 1%

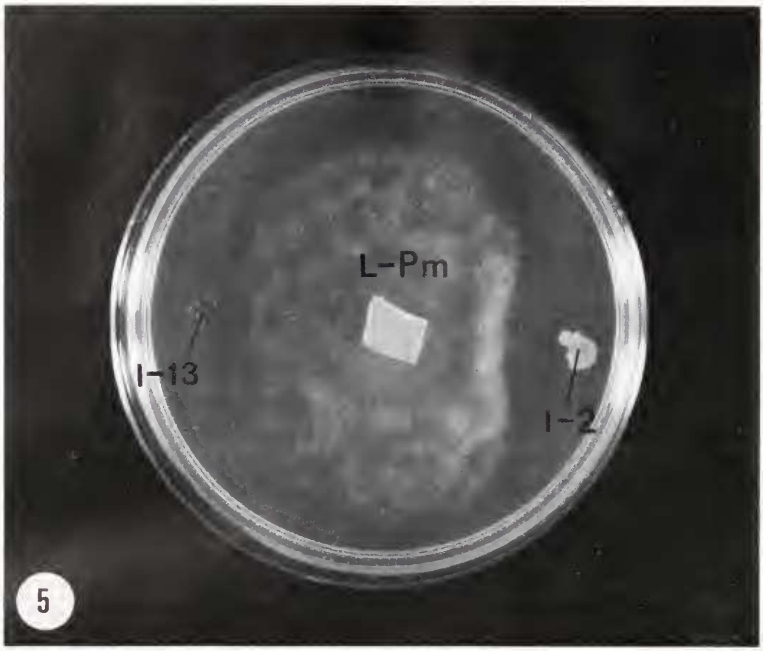
TABLE II

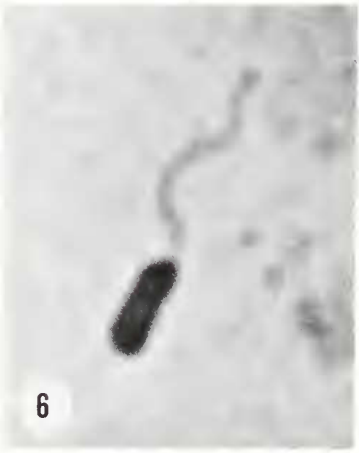
Growth rates of various fungal isolates on agar plates of different media, measured in millimeters of radial growth per day

Media	L-1	L-6	L-3b	L-815	CE	L-F2	L-Pm	H-2	H-222	S-At
1/3 MA	0.5	1.0	2.0	1.2	2.4	10.0	11.9	0	0	18.5
SIA	0.3	0.9	1.7	0.5	2.7	9.7	6.5	*	0.7	15.0
CMA		*	1.7	0.7	0.9	6.8	7.2	0	0	17.0
1% KCl-CMA		0	1.0	0.5	0.9	7.2	7.3	*	0	12.5
2.5% KCl-CMA		0	0.5	0.6	0.4	4.0	4.2	0	0	2.5
1% NaCl-CMA		0.7	2.3	1.6	2.3	9.5	9.2	0.7	1.3	12.0
2.5% NaCl-CMA		0.7	0.8	0.5	1.0	5.3	4.8	1.7	2.5	1.0
1/3 SW-CMA		0.6	2.4	1.5	1.8	10.2	10.5	0.7	1.6	14.5
1/2 SW-CMA	0.6	1.3	2.7	1.9	1.7	10.1	9.0	1.7	2.9	8.3
1/2 SW-CMAG	0.5	1.1	3.3	2.0	1.3	10.6	9.5	1.8	2.7	7.8

* Growth of less than 0.1 mm/day.

L-1: *Lagenidium callinectes* from *Callinectes sapidus* in North Carolina (Bland and Amerson, 1973).
L-6: *Lagenidium callinectes* from algae in Washington (by Gotelli in 1973).
L-3b: *Lagenidium callinectes* from *Penaeus setiferus* in Texas (Lightner and Fontaine 1973).
L-815: *Lagenidium callinectes* from *Penaeus stylirostris* in Mexico (by Lightner in 1976).
CE: *Lagenidium callinectes* from *Penaeus monodon* in the Philippines (by Gacutan in 1978).
L-F2: *Lagenidium callinectes* from *Pandalus platyceros* in California (by Fisher in 1975).
L-Pm: *Lagenidium callinectes* from *Palaemon macrodactylus* in California (described here).
H-2: *Haliphthoros milfordensis* from *Penaeus setiferus* in North Carolina (Tharp and Bland, 1977).
H-222: *Haliphthoros milfordensis* from *Homarus americanus* in California (Fisher *et al.*, 1975).
S-At: *Saprolegnia sp.* from *Acipenser transmontanus* in California (by Fisher in 1981).
Media are described in text.





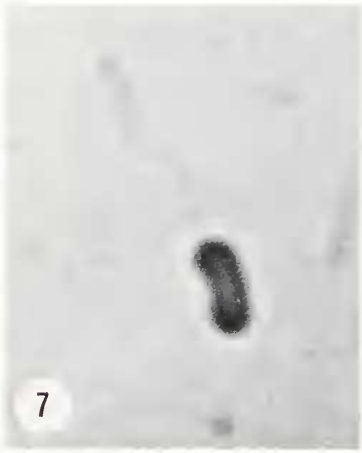
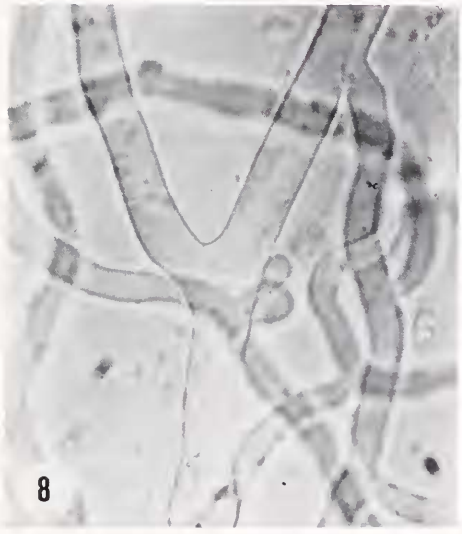
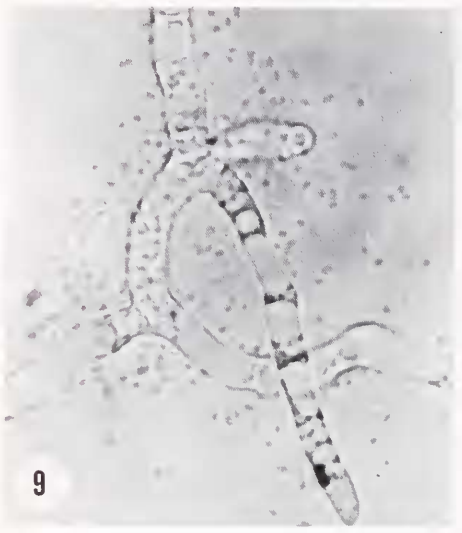


FIGURE 5. Bacterial isolates I-13 and I-2 on agar media (1/3 MA) restricted the growth of the fungus L-Pm growing from a fungal slab transfer in the center of the plate. I-13 and I-2 formed zones of inhibition of 4–8 mm and 6–10 mm respectively.

FIGURES 6 and 7. Flagella stain revealed both I-13 (Fig. 6) and I-2 (Fig. 7) had a single polar flagellum. Straight or slightly curved rods estimated at 2.2 × 0.6 μm with an extended flagellum of 4.4 μm, these marine bacteria are believed to belong to the genus *Alteromonas*. (8,250×).

NaCl concentration, and grow even faster with the balanced salts provided by 1/3 SW-CMA and 1/3 MA. Growth rates decrease as concentrations approach either fresh water or the salt equivalents of full-strength sea water (2.5% NaCl or KCl).





FIGURES 8 and 9. A small agar slab of growing L-Pm was placed in a drop slide (Fig. 8; 1,350×), then inoculated with I-2 bacteria and examined under a compound light microscope. After two days (Fig. 9; 1,340×), there were cavities formed in the hyphal cytoplasm. After six days (not shown), the fungal hyphae were completely devoid of cytoplasm. No stain.

Antagonistic effects of bacteria

There were 177 bacterial isolates obtained from the duplicate 10^{-2} dilution plates from embryo samples of fifteen females taken from their natural environment. Of these, 27 (15%) restricted fungal growth at least 1 mm from the edge of the bacterial colony. An additional 18 (10%) retarded fungal growth by slowing the rate or decreasing the density of the hyphae.

Two bacteria, I-2 and I-13, isolated from embryos on females held in the laboratory system, restricted growth of L-Pm on 1/3 MA plates (Fig. 5). Zones of inhibition were 6–10 mm and 4–8 mm, respectively. Growth of L-Pm from agar slabs in broth (1/3 MB) was luxuriant and easily observable after only 1 day, whereas there was no growth in broth inoculated with I-2 or I-13 bacteria. These bacteria are shown in Figures 6 and 7.

Fungal growth across Nalgene Nutrient Pad Kit membranes, hydrated with sterile 1/3 SW, was thick and luxuriant, reaching a 1–2 mm radius the first day and increasing to over 4 mm by the third day. Fungal slabs retrieved from the membrane, trimmed, and plated on 1/3 MA showed 6–10 mm growth in 24 hours. No growth, however, was recorded after 3 days by fungi on nutrient pads hydrated with broth culture of I-2 bacteria. Fungal slabs retrieved from this treatment after 1 day grew 6–8 mm when plated on 1/3 MA for 24 hours, but those retrieved after 2 days grew only 0–2 mm in 24 hours and those retrieved after 3 days did not grow even after 48 hours on 1/3 MA.

Figures 8 and 9 show the degradation of L-Pm by bacterial isolate I-2. Addition of the bacteria to the growing fungus appeared to cause cavity formation in the hyphal cytoplasm by 2 days (Fig. 9). Six days after the addition of bacteria, fungal hyphae were completely devoid of cytoplasm. This result was verified with stained

TABLE III

Metabolic characteristics of the bacteria I-2 and I-13 compared to recorded characterists of Alteromonas haloplanktis *strain 215*[1] *(A215) and* A. undina *strain 272*[2] *(A272)*

Metabolic characteristics	I-2	I-13	A215	A272
Utilizes:				
Adonitol	−	−	−	−
Citrate	+	+	+	−
Erythritol	−	−	−	−
Fructose*	+	+	+	−
Fumarate	+	+	+	+
Galactose	−	−	−	−
Glucose*	+	+	+	+
Inulin	−	−	−	−
L-Arabinose	−	−	−	+
Lactose	−	−	−	−
m-hydroxybenzoate	−	−	−	−
Maltose	+	−	+	+
Mannose	−	−	+	−
Melibiose	−	−	−	−
N-acetylglucosamine	+	+	+	+
Rhamnose	−	−	−	−
Sorbitol	−	−	−	−
Succinate	+	−	+	+
Sucrose*	+	+	+	+
Trehalose	+	+	−	+
Xylose	−	−	−	−
Produces:				
Alginase	−	−	−	−
Amylase	+	+	−	+
Chitinase	+	+	−	+
Gelatinase	+	+	+	+
Lipase	+	+	+	+

* Negative results for fermentation test.
[1] Baumann and Baumann, 1981.
[2] Chan *et al.*, 1978.

sections. Scanning electron microscopy revealed no visible damage to the exterior surface of the hyphal wall.

Characteristics of the bacteria

Characteristics of the bacterial isolates are shown in Table III. The Gram-negative, marine (requires Na^+), non-fermentative, straight or slightly curved rods of I-2 are motile and form glossy, circular, opaque colonies on 1/3 MA within 3 days at 25°C. I-13 shows similar characteristics except colonies are dark brown and adhere tightly to an agar surface. Both isolates produce extracellular enzymes, including amylase, gelatinase, lipase and chitinase, and each has a single polar flagellum (Figs.

TABLE IV

Distance in millimeters between colonies of I-2 bacteria and the nearest growth of L. callinectes *(strains L-6, L-3b, L-815, CE, and L-F2),* H. milfordensis *(H-2 and H-222),* Saprolegnia *(S-At) and the present isolate L-Pm plated on agar plates of different media*

Media	L-6	L-3b	L-815	CE	L-F2	L-Pm	H-2	H-222	S-At
1/3 MA	2	8	7–10	9	10	7			2
SIA	10	10	8–10	3–8	11	7			2
1/2 SW-CMA	5	4–8	8–9	9	1/0*	1/0*	2/0*	3/0*	2
1/2 SW-CMAG	5	4–9	5–10	9–10	1/0*	1/0*	3/0*	2/0*	2

* Fungal growth was initially inhibited (first number) but then gradually grew over the zone of inhibition to make contact with the bacterial colony (zone of inhibition then equals 0 mm, the second number).

Media are described in the text and fungal isolates are keyed in Table II.

6, 7). These characteristics place them among the marine heterotrophs of the genus *Alteromonas.*

Bacterium I-2 restricts or temporarily inhibits all the fungi tested (Table IV). L-Pm and L-F2 were restricted 7 and 11 mm away from the bacterial colony when plated on 1/3 MA or SIA. However, when they were plated on 1/2 SW-CMA or 1/2 SW-CMAG, only temporary inhibition occurred, slowing the fungal growth within 1 mm of the bacterial colony for a few days, but eventually allowing it to touch. Similar temporary inhibition of L-F2, L-Pm, and S-At was exhibited by *Alteromonas* strains 261, 272, 215, 107 and 113 on 1/3 MA. No inhibition was shown by *Alteromonas* strains 40, 121, and 8 against these same fungi. The presumptive *Alteromonas* isolates, I-2 and I-13, restricted fungal growth several millimeters from their respective colonies (Fig. 5).

DISCUSSION

Externally brooded eggs of *P. macrodactylus* were found susceptible to a fungal pathogen presumptively identified as *Lagenidium callinectes.* Infection always resulted in death, but was observed only on eggs detached from the female or on eggs attached to females with excised first pereiopods. This implied that the first pereiopods of the female played an important role in the defense against fungal infection, either by brushing away fungal propagules or by removing infected eggs to protect healthy eggs in the clutch. Cleaning by the pereiopods has also been shown to reduce bacterial association with the egg surfaces (Fisher, 1983). In a similar study, Bauer (1979) found a higher incidence of particulate debris, bacteria, nematodes, hypotrichous ciliates and dead eggs in clutches of the caridean *Heptacarpus pictus* when the first pereiopods were excised.

Fertilized eggs in this study were less susceptible to fungal infection than unfertilized eggs. This may have been the result of a hardened outer embryonic coat, the fertilization membrane, formed during the fertilization reaction (Cheung, 1966). The harder coat may resist fungal penetration and thereby limit infection. In addition, hyphae seemed unable to penetrate mature (10- to 12-day-old) embryos and, instead, formed a mat around them. Death in this case was probably caused by asphyxiation rather than tissue destruction. Lack of penetration in these older embryos may also be due to increased hardness of the outer embryonic coat. Burkenroad (1947) found increasing resistance to cold, concentrated HCl in the outer coat

of developing *Palaemonetes vulgaris* embryos and Cheung (1966) claimed that the egg coat of *Astacus pallipes* became considerably harder in older embryos.

Lagenidium infection of adult *P. macrodactylus* occurred in the holding system and resembled *Haliphthoros milfordensis* infection of *Homarus* postlarvae (Fisher *et al.*, 1975) and *Aphanomyces astaci* infection of European crayfish (Unestam and Weiss, 1970). Several sites of infection were observed, but normally in areas of flexible or "soft" exoskeleton (ventral abdomen, joints). Melanization of infected areas occurred in an apparent attempt to restrict the penetration of the fungus. Molting or removal of infected tissue could halt progress of the disease. Fungal propagules can exist in the system without infecting either adults or eggs, infection presumably requiring a portal of entry and/or sufficient nutrient to allow the growth necessary for penetration.

Growth rate and salt concentration comparisons support the presumptive identification of the fungus as *Lagenidium callinectes* (Table II). L-Pm is unquestionably similar to L-F2, a confirmed member of the species and a previous isolate from northern California. The two isolates have similar salt optima (which also compare favorably with earlier studies of L-F2 by Nilson *et al.*, 1976) and are similarly inhibited by the bacterium I-2 (Table IV).

Bacterial isolates I-2 and I-13 are heterotrophic marine bacteria that produce the enzymes amylase, lipase, gelatinase and chitinase (Table III). *Alteromonas* are the only known heterotrophic marine eubacteria that produce extracellular enzymes (Baumann and Baumann, 1981) and *A. haloplanktis* and *A. undina*, two species from northern California, closely resemble the metabolic characteristics thus far determined for I-2 and I-13 (Table III). Tested strains of *A. haloplanktis* and *A. undina* did not, however, inhibit fungal growth with the same potency of I-2 or I-13.

Bacterial isolate I-2 inhibited growth on agar plates of several strains of *L. callinectes* and *H. milfordensis*, two pathogens of marine Crustacea (Table IV). It also inhibited a freshwater fungus (*Saprolegnia sp.*) isolated from infected sturgeon eggs (*Acipenser transmontanus*). *Saprolegnia* has been recognized as a pathogen for a wide range of fish species (Neish and Hughes, 1980), including the commercially important salmonids, cichlids and anguillids. Successful isolation and characterization of the active substance produced and released by this bacterial isolate may substantially aid in the artificial culture of marine crustaceans and freshwater fishes.

Fungal inhibition has been demonstrated by I-2 on agar plates and on nutrient pads across filter membranes, indicating that the active agent is extracellular. Chitinase was released extracellularly by both I-2 and I-13 and, since it was found to aid in lysing the cell wall of the fungus *Aspergillus* (Horikoshi and Iida, 1959), it may play a role in the antifungal action. Other species of *Alteromonas* which release chitinase did not, however, significantly restrict the growth of L-Pm. Nor did scanning electron microscopy reveal any external deterioration of the fungal wall after incubation with I-2.

The ecological significance of antifungal bacteria inhabiting the surfaces of externally brooded embryos of *P. macrodactylus* has not been investigated. Bacterial type I-2 was isolated during an experiment where penicillin-treated embryos, exhibiting high counts of penicillin-tolerant bacteria, contracted an infection of L-Pm. I-2 was isolated from the untreated, uninfected control embryos and found to be both antifungal and susceptible to penicillin. This implied that I-2 or similar bacteria were protecting the embryos from fungal infection. The high percentage (25%) of bacteria from nature with antagonistic properties supports this possibility and the

potent effects of I-2 on a variety of fungi indicates that antifungal bacteria may have a wide range of influence.

The results of this study demonstrate the importance of the cleaning chelipeds in protecting embryos against fungal infection. How decapods without the ability to preen their brooding embryos (the Dungeness crab, *Cancer magister,* for example) protect them from fungal infection is not known. Susceptibility to fungal infection, unlike susceptibility to bacterial infestation (Fisher, 1983), is affected by fertilization and embryonic age. Moreover, the presence of antifungal bacteria on egg surfaces opens the possibility of a wide range of microbial actions and interactions that could affect the survival of brooding embryos.

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LITERATURE CITED

ABRAHAMS, D., AND W. D. BROWN. 1977. Toxicity to juvenile European lobster (*Homarus gammarus*) of several anti-fungal agents used to control *Haliphthoros milfordensis*. *Aquaculture* **12:** 31–40.

AIYER, R. P. 1949. On the embryology of *Palaemon idae* Heller. *Proc. Zool. Soc. Bengal* (Calcutta) **2**(2): 101–147.

ANDERSEN, F. J., M. S. WOLFE, AND D. J. FAULKNER. 1974. Autotoxic antibiotic production by a marine *Chromobacterium*. *Mar. Biol.* **24**(4): 281–285.

ARMSTRONG, D. A., D. V. BUCHANAN, AND R. S. CALDWELL. 1976. A mycosis caused by *Lagenidium sp.* in laboratory reared larvae of the Dungeness crab, *Cancer magister,* and possible chemical treatments. *J. Invertebr. Pathol.* **28:** 329–336.

BALLESTER, M., J. M. BALLESTER, AND J. P. BELAICH. 1977. Isolation and characterization of a high molecular weight antibiotic produced by a marine bacterium. *Microb. Ecol.* **3:** 289–303.

BAUER, R. T. 1979. Antifouling adaptations of marine shrimp (Decapoda: Caridea): gill cleaning mechanisms and grooming of brooded embryos. *Zool. J. Linn. Soc.* **65:** 281–303.

BAUMANN, P., AND L. BAUMANN. 1981. The marine gram-negative eubacteria: Genera *Photobacterium, Beneckea, Alteromonas, Pseudomonas* and *Alcaligenes*. Pp. 1302–1331 in *The Prokaryotes* Vol II, M. P. Starr, H. Stolp, H. G. Truper, A. Balows and H. G. Schlegel, eds. Springer-Verlag, New York, Heidelberg, Berlin.

BLAND, C. E., AND H. V. AMERSON. 1973. Observations on *Lagenidium callinectes:* isolation and sporangial development. *Mycologia* **65:** 310–320.

BLAND, C. E., D. G. RUCH, B. R. SALSER, AND D. V. LIGHTNER. 1976. Chemical control of *Lagenidium,* a fungal pathogen of marine Crustacea. *Proc. World Mariculture Soc.* **7:** 445–472.

BURKENROAD, M. D. 1947. Reproductive activities of decapod Crustacea. *Am. Nat.* **81:** 392–398.

BURKHOLDER, P. R., R. M. PFISTER, AND F. H. LEITZ. 1966. Production of a pyrrole antibiotic by a marine bacterium. *Appl. Microbiol.* **14**(4): 649–653.

CHAN, K. Y., L. BAUMANN, M. M. GARZA, AND P. BAUMANN. 1978. Two new species of *Alteromonas: Alteromonas espejiana* and *Alteromonas undina*. *Int. J. Syst. Bacteriol.* **28**(2): 217–222.

CHEUNG, T. S. 1966. The development of egg-membranes and egg attachment in the shore crab, *Carcinus maenas,* and some related decapods. *J. Mar. Biol. Assoc. U. K.* **46:** 373–400.

COOK, H. L. 1971. Fungi parasitic on shrimp. *FAO Aquacult. Bull.* **3:** 13.

COUCH, J. N. 1942. A new fungus on crab eggs. *J. Elisha Mitchell Sci. Soc.* **58:** 158–164.

DELVES-BROUGHTON, J. 1974. Preliminary investigations into the suitability of a new chemotherapeutic, Furanace, for the treatment of infectious prawn diseases. *Aquaculture* **3:** 175–185.

DEWEL, W. C., AND W. H. CLARK, JR. 1972. An ultrastructural investigation of spermiogenesis and the mature sperm in the anthazoan *Bunodosoma cavernata*. *J. Ultrastruct. Res.* **40:** 417–431.

FISHER, W. S. 1983. Eggs of *Palaemon macrodactylus:* II. Association with aquatic bacteria. *Biol. Bull.* **164:** 201–213.

FISHER, W. S., E. H. NILSON, AND R. A. SHLESER. 1975. Effect of the fungus *Haliphthoros milfordensis*

on the juvenile stages of the American lobster *Homarus americanus*. *J. Invertebr. Pathol.* **26**: 41–45.

FISHER, W. S., E. H. NILSON, L. F. FOLLETT, AND R. A. SHLESER. 1976. Hatching and rearing lobster larvae (*Homarus americanus*) in a disease situation. *Aquaculture* **7**: 75–80.

GAUTHIER, M. J. 1976. *Alteromonas rubra* sp. nov., a new marine antibiotic-producing bacterium. *Int. J. Syst. Bacteriol.* **26**: 459–466.

GAUTHIER, M. J. 1977. *Alteromonas citrea*, a new gram-negative, yellow-pigmented bacterium isolated from seawater. *Int. J. Syst. Bacteriol.* **27**: 349–354.

GAUTHIER, M. J., AND V. A. BREITTMAYER. 1979. A new antibiotic-producing bacterium from seawater: *Alteromonas aurantia* sp. nov. *Int. J. Syst. Bacteriol.* **29**: 366–372.

GAUTHIER, M. J., AND G. N. FLATAU. 1976. Antibacterial activity of marine violet-pigmented *Alteromonas* with special reference to the production of brominated compounds. *Can. J. Microbiol.* **22**: 1612–1619.

HORIKOSHI, K., AND S. IIDA. 1958. Lysis of fungal mycelia by bacterial enzymes. *Nature* **181**: 917–918.

HORIKOSHI, K., AND S. IIDA. 1959. Effect of lytic enzyme from *Bacillus circulans* and chitinase from *Streptomyces sp.* on *Aspergillus oryzae*. *Nature* **183**: 186–187.

LIGHTNER, D. V., AND C. T. FONTAINE. 1973. A new fungus disease of the white shrimp *Penaeus setiferus*. *J. Invertebr. Pathol.* **22**: 94–99.

MAYFIELD, C. I., AND W. E. INNISS. 1977. A rapid simple method for staining bacterial flagella. *Can. J. Microbiol.* **23**: 1311–1313.

NEISH, G. A., AND G. C. HUGHES. 1980. Book 6: Fungal diseases of fishes. Pp. 1–159 in *Diseases of Fishes*, S. F. Snieszko and H. R. Axelrod, eds. T.F.H. Publications Inc., Neptune, New Jersey.

NEWMAN, W. A. 1963. On the introduction of an oriental shrimp (Caridea, Palaemonidae) to San Francisco Bay. *Crustaceana* **5**: 119–132.

NILSON, E. H., W. S. FISHER, AND R. A. SHLESER. 1976. A new mycosis of larval lobster (*Homarus americanus*). *J. Invertebr. Pathol.* **27**: 177–183.

SPURR, A. R. 1969. A low viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.* **36**: 31–43.

THARP, T. P., AND C. E. BLAND. 1977. Biology and host range of *Haliphthoros milfordensis* Vishniac. *Can. J. Bot.* **55**: 2936–2944.

UNESTAM, T., AND D. W. WEISS. 1970. The host-parasite relationship between freshwater crayfish and the crayfish disease fungus *Aphanomyces astaci*: responses to infection by a susceptible and a resistant species. *J. Gen. Microbiol.* **60**: 77–90.